

## Development of Immunity by the Tomato Clownfish *Amphiprion frenatus* to the Dinoflagellate Parasite *Amyloodinium ocellatum*

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**Abstract.**—The dinoflagellate *Amyloodinium ocellatum*, which causes amyloodiniosis or “marine velvet disease,” is one of the most serious ectoparasitic diseases affecting warmwater marine fish culture worldwide. We demonstrated that tomato clownfish *Amphiprion frenatus* can develop strong immunity to infection following repeated nonlethal parasitic challenges. The protective response is long-lived and directed against the trophont stage of the parasite.

*Amyloodinium ocellatum* is an ectoparasitic dinoflagellate with a triphasic life cycle consisting of infectious dinospore, attached-feeding trophont, and reproducing tomon stages. It is found in warmwater marine and estuarine environments worldwide. Serious problems have not been documented in natural environments, but when fish populations are crowded or otherwise stressed, as in mariculture or the ornamental fish trade, the parasite can rapidly increase in numbers and cause high mortality (Noga and Levy 1995). Although several fish species appear to be resistant or to develop resistance to *A. ocellatum* infection (Lawler 1977, 1980; Paperna 1980), the dynamics of this resistance have not been characterized. The tomato clownfish *Amphiprion frenatus*, a damselfish of the Indopacific region, is very susceptible to *Amyloodinium* infection.

This report describes the development of immunity by the tomato clownfish to lethal challenge by *Amyloodinium ocellatum* and the persistence of immunity for several months in the absence of antigenic stimulation. Our data indicate that the protective immune response is directed against the trophont stage of the parasite.

### Methods

**Maintenance of organisms.**—*Amyloodinium ocellatum* (isolate 85-1 of DC-1) was maintained in cell culture by using the methods developed by Noga (1987, 1989). Aquarium-reared tomato

clownfish (4–13 g, 47–71 mm total length) were obtained from Aqualife Resources (Fort Lauderdale, Florida). Fish were maintained in 150-L round fiberglass aquaria containing 25‰ artificial seawater (ASW). Prior to all *A. ocellatum* experiments, copper levels were maintained between 0.15 and 0.20 mg/L to control ectoparasites. Parasite returns from fish handled according to this protocol or fish maintained free of copper were similar.

**Sublethal challenge of fish with *A. ocellatum*.**—Cell-cultured tomons were incubated in wells of a 24-well cell culture plate with a modified sterile ASW mixed with Hanks' balanced salt solution (IO2/HBSS) described by Noga (1989). To obtain parasites of approximately the same age, dinospores that excysted within a single 24-h period (the peak of infectivity) were gently aspirated from the top of a well via a 5-mL pipette; care was taken to not aspirate tomons, which were settled on the bottom. Dinospores were then transferred to a sterile, 15-mL centrifuge tube. A sample was stained with Lugol's iodine and counted by using an inverted-phase contrast microscope.

Fish were routinely exposed to a sublethal challenge of 40,000 dinospores/fish at a concentration of 200 dinospores/mL. Equal numbers of fish were placed in an identical container without dinospores as a control. After 30 min, all fish were gently removed from each container and placed in separate 38-L holding aquaria with 25‰ ASW to allow the *A. ocellatum* trophonts to mature. After 3 d, each fish was dipped in distilled water (dH<sub>2</sub>O) for 3 min to remove the trophonts for counting;

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then fish were placed in a recovery aquarium containing 0.15–0.20 mg copper/L.

Infection rates were calculated as percent parasite recovery rate (PRR):

$$\frac{\text{number of tomites recovered/fish} \times 100}{\text{number of dinospores/fish in challenge}}$$

Individuals that exhibited parasite recovery rates of less than 1% were considered immune.

In a separate experiment, both immune and non-immune fish were exposed to 40,000 dinospores/fish, then dipped in dH<sub>2</sub>O at 12, 24, or 48 h post-infection.

*Collection of fish-propagated parasites for challenge studies.*—Parasites were propagated on fish as described by Bower et al. (1987) with the following modifications. Individual infected fish were placed in cups of 60 mL dH<sub>2</sub>O. After 3 min, fish were removed and 60 mL of 50‰ ASW was immediately placed into each cup, raising the salinity to 25‰. Trophonts were allowed to settle for 15 min. Then 100 mL of ASW was replaced with new 25‰ ASW. The trophonts were washed similarly four more times and then pipetted into wells of a 24-well cell culture plate. The trophonts in each well were gently mixed, and three 50- $\mu$ L aliquots from each well were placed in separate wells of a 96-well plate for counting (Bower et al. 1987). The trophonts remaining in the 24-well plate were then allowed to settle for 5–10 min. The ASW was gently aspirated and gently replaced with 25‰ ASW containing 100  $\mu$ g of streptomycin sulfate and 100 units of penicillin/mL. Trophonts were allowed to settle for 10 min and this washing procedure was repeated five more times. Dividing tomites from individual fish were kept in the 24-well plate at 26°C for 4 d, at which time excysted dinospores were counted and used in the following experiments.

*Infectivity of cell-cultured versus fish-cultured A. ocellatum dinospores.*—By counting the number of trophonts recovered after a sublethal challenge as described above, we compared the infectivity of dinospores that had been propagated in cell culture for more than 10 years with fish-cultured dinospores that had been passed for at least two generations on fish.

In a separate experiment, on days 0, 7, 14, and 28, ten individually tagged fish were challenged with 40,000 cell-cultured dinospores/fish; trophonts were later harvested and counted. Also on days 0, 7, 14, and 28, ten fish that had not been previously experimentally exposed to *Amyloodi-*

*nium ocellatum* (previously unexposed) were also challenged as a control.

*Lethal challenge experiments.*—Five immune and five unexposed fish were placed in separate 9.5-L aquaria. Fish-cultured dinospores were added to each aquarium (200,000/fish), and fish were observed daily for signs of morbidity or mortality.

To determine if resistance was long-lived, three groups of five fish were challenged with 40,000 dinospores/fish in separate 9.5-L aquaria. The parasites were allowed to go through their normal life cycle on fish in the challenge aquaria. Group 1 consisted of previously unexposed fish. These fish were replaced with new unexposed fish as mortality occurred so that the parasite could continue to propagate. Five new fish were added on day 4 and seven on day 8. Group 2 consisted of fish which had been previously challenged until immune but had not been challenged within the last 3 months. Group 3 consisted of immune fish as in group 2 but these fish had not been challenged within the last 6 months. All three groups were observed daily for morbidity or mortality for 12 d.

*Dinospore attachment assay.*—The ability of *A. ocellatum* dinospores to attach to fish was evaluated with a dinospore attachment assay. To each of eighteen 600-mL beakers was added 400 mL of a dinospore suspension (140 dinospores/mL). A water sample was taken from each beaker and counted to confirm the number of motile dinospores in each beaker. One fish was placed in each of 12 beakers for 1 h (six immune fish and six previously unexposed fish). Six beakers without fish were included as controls. At the end of the 1-h challenge, fish were removed and samples were again taken from all beakers and counted.

In a separate experiment, five immune and five previously unexposed fish were infected with 40,000 dinospores/fish, then dipped in dH<sub>2</sub>O at 12, 24, and 48 h postinfection.

*Statistical analysis.*—Means, standard deviations, and Student's *t*-test with *P*-values were calculated by using Microsoft Excel for Windows.

## Results

### *Infectivity of Cell-Cultured versus Fish-Cultured A. ocellatum Dinospores*

To compare infectivity of in vitro cultured versus fish-cultured *A. ocellatum*, parasite recovery rates of fish sublethally challenged with parasites from each source were analyzed (*N* = 10 fish for each group). Fish sublethally challenged with cell-cultured *A. ocellatum* had a parasite recovery rate

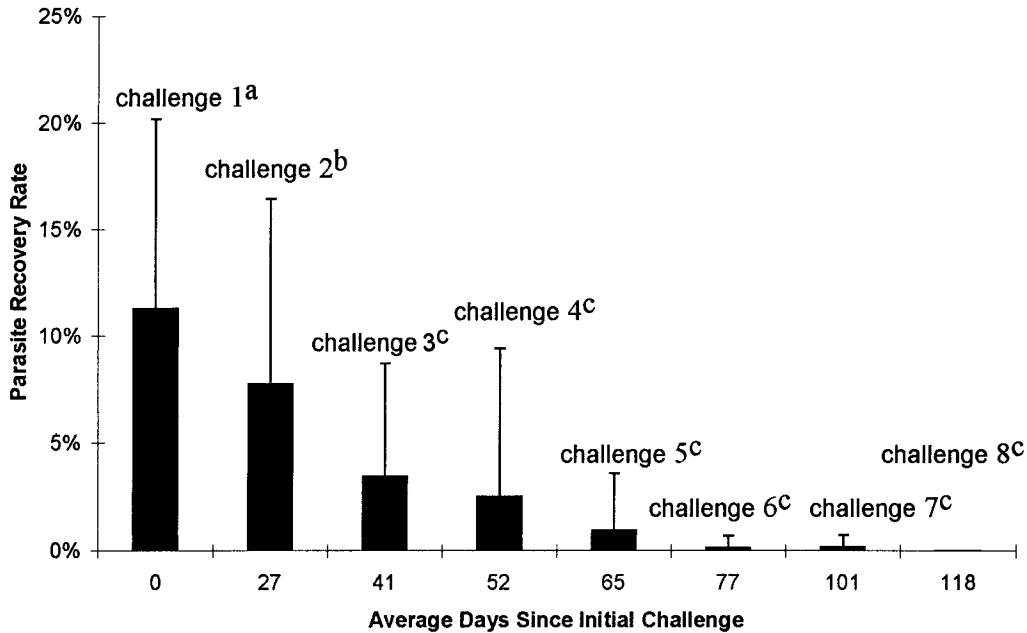


FIGURE 1.—Parasite recovery rate during standard challenge of tomato clownfish. Data representing 507 challenges were analyzed for parasite recovery rate from individual fish. For challenges 1–8,  $N = 161, 106, 102, 72, 39, 19, 6,$  and 2 fish, respectively. The ranges of days from first challenge to each subsequent challenge were as follows: to second challenge, 6–80 d; to third challenge, 14–90 d; to fourth challenge, 23–108 d; to fifth challenge; 35–107 d; sixth challenge = 48–115 d; to seventh challenge, 63–131 d; and to eighth challenge, 108–128 d. Values are means + SDs. Values with different superscript letters are significantly different ( $P < 0.05$ ) from each other.

of 9.1% ( $\pm 3.5$ ), somewhat less than fish challenged with fish-cultured parasites, which had a PRR of 13.1% ( $\pm 4.0$ ;  $P = 0.014$ ). Dinospores from fish-cultured *A. ocellatum* were used for subsequent experiments.

#### *Development of Immunity during Standard Challenges*

While performing parasite challenges, we noticed that it was very difficult to recover *A. ocellatum* trophonts from fish that had been repeatedly challenged, indicating that fish became resistant. Evaluation of these results showed that mean PRRs declined from 11.3% after one challenge to 7.8% after two challenges ( $P = 0.0006$ ) to 3.5% after three challenges ( $P < 0.0006$ ). Mean PRRs in challenges 3–8 were not significantly different (Figure 1).

To determine the time or number of challenges required for development of immunity, a weekly sublethal challenge experiment was performed. The PRR on days 0 and 7 averaged about 20% for both experimental and control fish ( $N = 10$  for each group). During the third challenge with *A. ocellatum* dinospores (14 d after the initial chal-

lenge), the sequentially challenged fish began to show resistance (PRR = 4%), whereas initially challenged fish remained highly susceptible (PRR = 19%). By the fourth challenge (day 28), the sequentially challenged fish had a PRR of less than 1% (Figure 2).

#### *Immune Protection from Lethal Challenge*

Preliminary data demonstrated that challenging a tomato clownfish (6 g, mean weight) with 200,000 dinospores was lethal whereas a challenge with 40,000 dinospores/fish was consistently non-lethal. Fish that had previously exhibited a PRR of less than 1% showed no morbidity or mortality after challenge with 200,000 dinospores/fish; in contrast, all previously unexposed fish died within 5 d ( $N = 10$  fish/group).

In the experiment to determine if resistance induced by sublethal challenge conferred long-term protection, four of five previously unexposed fish died by day 4, and the surviving fish as well as seven added fish died by day 12. None of the previously challenged fish that had not been challenged during the prior 3 months or 6 months displayed morbidity.

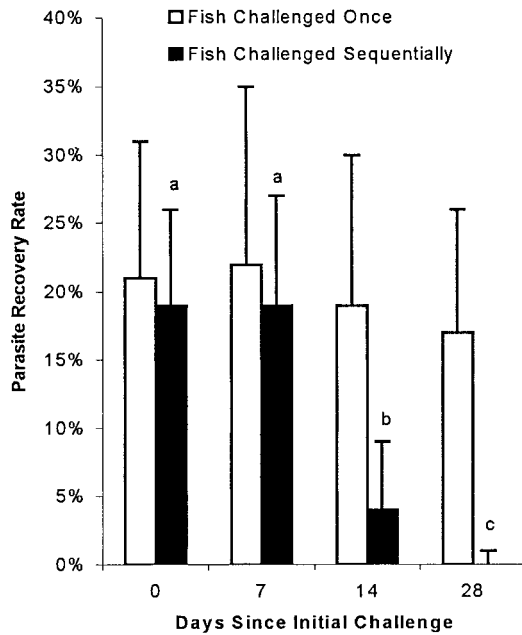


FIGURE 2.—Development of immunity following weekly challenge with *A. ocellatum* dinospores. Values are means + SD;  $N = 10$  for each group. Values for the sequentially challenged fish with different letters above the bars are significantly different ( $P < 0.05$ ) from each other.

#### *Effect of Immunity on In Vivo Parasite Attachment and Survival*

After a 1-h exposure, the numbers of dinospores in beakers containing immune or previously unexposed fish were statistically the same ( $P > 0.5$ ): beakers with immune fish had 36.3% ( $\pm 10.0$ ) of the dinospores remaining in the artificial seawater, whereas beakers with previously unexposed fish had 32.0% ( $\pm 12.6$ ) of the original number. Control beakers with no fish had 98% of the original number of dinospores. The PRR of immune fish was 0%, whereas previously unexposed fish had a mean PRR of 10.6% ( $\pm 4.0$ ).

In the second experiment, at 12 h, trophonts were recovered from immune fish but not from previously unexposed fish. At 24 h, trophonts were recovered from previously unexposed fish but not from immune fish. This finding indicates that the immune response appears to be associated with the early rejection (<24 h) of trophonts.

#### Discussion

We provide clear evidence that fish can develop strong resistance to infection by *Amyloodinium ocellatum* after repeated sublethal challenges. Results from more than 123 individually tagged fish

in more than 600 challenges showed that more than 100 of them developed immunity after 3–4 sublethal challenges (Figure 1). These results closely parallel studies of *Ichthyophthirius multifiliis*, which showed that at least some fish species can develop immunity to this ciliated protozoan through sublethal challenge (Hines and Spira 1974; Goven et al. 1980; McCallum 1986). Weekly challenges by *A. ocellatum* performed on the same fish showed that significant immunity was exhibited by some fish 2 weeks after the initial challenge ( $P = 0.037$ ); by the fourth challenge on day 28, trophonts were recovered only from one fish, which had a PRR of 1.2%. The kinetics of this response suggest a secondary immune response. The involvement of antibody was very strongly indicated by a protective memory response that was present for at least 6 months.

The second lethal challenge experiment was meant to simulate what might occur in intensive mariculture where fish are closely confined and *A. ocellatum* can rapidly multiply to lethal densities. When fish were previously unexposed and, therefore, highly susceptible, parasites not only killed the initial hosts but remained present in sufficient numbers to kill fish introduced later. In contrast, parasites were unable to cause mortality or obvious morbidity among resistant fish. Additionally, as dinospores initially attach to both immune and nonimmune fish (see below), the presence of immune fish might provide “herd immunity” by decreasing the parasite load in the environment, allowing more time for previously unexposed fish to develop resistance.

Although we did not perform any experiments aimed at determining the specificity of the immune response, an unexpected outbreak of *Cryptocaryon irritans*, a skin-dwelling ciliate parasite, was equally lethal to both *A. ocellatum*-resistant and -susceptible clownfish (Cobb, unpublished data). This strongly suggests that the observed resistance was parasite-specific.

Experiments comparing dinospores propagated in cell culture with those cultured on fish showed that although our cell-cultured *A. ocellatum* isolate had not been exposed to fish for more than 10 years, it still retained significant infectivity and lethality.

Dinospore attachment to immune versus previously unexposed fish was not different. This result coupled with no observable differences in dinospore motility when exposed to immune and previously unexposed fish serum (Cobb, unpublished data) suggests that immunity is not directed to-

wards preventing dinospore attachment to the fish. An antitrophont mechanism is suggested by the observation that some very small trophonts could be removed from immune fish by the deionized water dip at 12 h, whereas no trophonts could be removed from previously unexposed fish until 24–36 h postinfection by this method. Thus dinospore attachment occurs equally well on immune and previously unexposed fish, but immune fish can reject trophonts or at least severely retard trophont development, suggesting that immunity is directed against trophonts rather than dinospores. Evidence that this response may be mediated through a specific antiparasite antibody has been collected (Cobb et al., in press).

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